

## PCT

**WORLD INTELLECTUAL PROPERTY ORGANIZATION**  
**International Bureau**

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : A61K 47/42, 37/14, 33/00, C12N 5/00, C07K 17/02		A1	(11) International Publication Number: WO 94/22482
		(43) International Publication Date: 13 October 1994 (13.10.94)	
(21) International Application Number: PCT/US94/03116		(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 23 March 1994 (23.03.94)			
(30) Priority Data: 08/037,289 26 March 1993 (26.03.93) US		Published With international search report.	
(71) Applicant: BIORELEASE TECHNOLOGIES, INC. [US/US]; 8 Industrial Way, Salem, NH 03079 (US).			
(72) Inventors: DiSORBO, Dennis, M.; 4 Chagnon Lane, Hudson, NH 03051 (US). REEVES, R., Bruce; 754 Straw Hill Road, Manchester, NH 03104 (US).			
(74) Agent: GATES, Edward, R.; Wolf, Greenfield & Sacks, 600 Atlantic Avenue, Boston, MA 02210 (US).			
(54) Title: COMPOSITIONS INCLUDING HEME-CONTAINING PROTEINS AND METHODS RELATING THERETO			
(57) Abstract			
<p>Heme-containing proteins having bound thereto nonoxygen gas ligands for <i>in vivo</i> and <i>in vitro</i> uses are provided. Cell culture preparations and pharmaceutical preparations, as well as methods for preparing the foregoing, also are provided. The preferred nonoxygen gas ligands are carbon dioxide, carbon monoxide and nitric oxide.</p>			
<p>Applicants: David J. Pinsky et al. Serial No.: 10/679,135 Filed: October 3, 2003 Exhibit 14</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

COMPOSITIONS INCLUDING HEME-CONTAINING PROTEINS  
AND METHODS RELATING THERETO

Field of the Invention

The invention relates to heme-containing proteins having bound thereto nonoxygen gas ligands for in vitro and in vivo uses, as well as cell culture preparations and pharmaceutical preparations relating thereto.

Background of the Invention

Cells grown under aerobic conditions can convert one molecule of glucose to carbon dioxide and water and generate 36 molecules of ATP. Under anaerobic conditions, however, one molecule of glucose is converted to lactic acid with the generation of only two molecules of ATP. Thus, oxygen depletion, or limitation, requires up to 18 times more glucose to be supplied to a cell to generate an equivalent number of ATP molecules produced under aerobic conditions. An efficient mechanism known for delivery of oxygen to organs is through the association of oxygen with hemoglobin.

Native human hemoglobin is predominantly a tetrameric molecule with a molecular mass of 64,000 daltons. The tetramer is composed of two pairs of alpha and beta sub-units held together by non-covalent forces. Each sub-unit (16,000 daltons) is conjugated to one heme moiety, and in the tetrameric configuration, each forms a heme pocket which maintains the iron atom in the ferrous state (a gas ligand binding state.) When hemoglobin is removed from the red blood cell the ferrous atom becomes susceptible to oxidation and conversion to the ferric state. This conversion results in the formation of methemoglobin, a form of hemoglobin that does not bind oxygen or other gas ligands and has a reduced solubility in solution.

SUBSTITUTE SHEET

-2-

Various investigators have attempted to purify, stabilize, and modify hemoglobin so that it may be used as a red blood cell substitute. Investigators have cross-linked hemoglobin intermolecularly and intramolecularly to avoid renal toxicity. In particular, when free hemoglobin is administered to a patient, it tends to dissociate into dimeric and monomeric units. These units are small enough such that they are filtered by the kidney and produce renal toxicity. By cross linking the sub-units of hemoglobin and polymerizing tetramers of hemoglobin, the dissociation of the sub-units and renal toxicity are avoided.

Cross-linking is not always desired because cross-linking techniques can involve multiple steps, can use potentially toxic reagents, and can adversely affect the affinity of oxygen or other gas ligands for the heme group. Nevertheless, the use of noncross-linked hemoglobin is not generally practiced because of the toxicity associated with dimeric and monomeric units.

When culturing cells in vitro, oxygen typically is applied to the cells by bubbling oxygen into the media in which the cells are suspended. Although it has been suggested that oxygen be supplied to cells by using medium containing hemoglobin saturated with oxygen, such attempts have been largely unsuccessful. Perhaps the lack of success is caused by the problems relating to the stability of hemoglobin.

Recently, investigators have saturated hemoglobin with carbon monoxide (CO), replacing the oxygen at the heme moiety, to stabilize hemoglobin against conversion to methemoglobin. For various reasons, the carbon monoxide then would be replaced with oxygen prior to use. Carbon monoxide has been known to act as a pulmonary poison in living organisms. In addition, carbon monoxide (and nitric oxide - NO) readily displace oxygen from hemoglobin. It, therefore, would be expected that once carbon monoxide or nitric oxide has been complexed to hemoglobin, that hemoglobin should not

SUBSTITUTE SHEET

-3-

readily act as an effective exchange agent for free oxygen in solution and should not readily increase available oxygen to cells or living tissues since hemoglobin affinity for CO and NO is substantially higher than that of oxygen. Furthermore, if the purpose of adding hemoglobin to cell culture or to blood is to enhance delivery of oxygen to cells or living tissue, then it only makes sense to preload the hemoglobin with oxygen. Thus, the clear teaching of the prior art is to replace carbon monoxide with oxygen prior to use.

The prior art also teaches that it is desirable to lower the binding affinity of hemoglobin for oxygen to promote the release of oxygen to cells or tissues. One example is the pyridoxalation of hemoglobin for the purpose of making hemoglobin a more effective oxygen delivery agent in vivo. In addition, cross-linking can improve the stability of hemoglobin.

It is known that oxygen, carbon monoxide, carbon dioxide, and nitric oxide bind to hemoglobin.

It is an object of the invention to provide methods and products for delivering nonoxygen gas ligands to cells and/or tissues.

It is an object of the invention to provide improved methods and products for culturing cells.

It is an object of the invention to provide methods for delivering nonoxygen gas ligands to cells for therapeutic purposes and for improved cell culture purposes.

These and many other objects will be understood with reference to the following description of the invention.

#### Summary of the Invention

The invention utilizes heme-containing proteins having bound thereto nonoxygen gas ligands for in vitro and in vivo uses, as well as cell culture preparations and pharmaceutical preparations relating thereto.

According to one aspect of the invention, a method for delivering a nonoxygen gas ligand to a cell is provided. The

**SUBSTITUTE SHEET**

-4-

environment of the cell is contacted with a nonoxygen gas ligand that is reversibly bound to a heme moiety of a heme-containing protein for the purpose of delivering the nonoxygen gas ligand to the cell. Preferably, the cell is contacted with a gas ligand selected from the group consisting of NO, CO, and CO<sub>2</sub>, and preferably the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

The heme-containing protein may be delivered in solution or may be contained in a liposome. Likewise, the heme-containing protein may be contained in a pharmaceutically acceptable carrier or in fresh cell culture medium.

It is preferred that the nonoxygen gas ligands occupy at least 25% of the heme moieties of the heme-containing protein, more preferred that the nonoxygen gas ligands occupy at least 50% of such moieties, even more preferred that the nonoxygen gas ligands occupy at least 75% of such moieties, and most preferred that the nonoxygen gas ligands saturate the heme moieties, occupying at least 90% of such moieties.

According to another aspect of the invention, a pharmaceutical preparation is provided. The pharmaceutical preparation is a mixture of a pharmaceutically acceptable carrier and a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen binding heme moieties of isolated heme containing protein. The preferred heme-containing proteins and gas ligands are as described above. Also as described above, the heme-containing protein may be contained in liposomes. The preferred concentration of the gas ligand also is as described above.

According to another aspect of the invention, a method for forming a pharmaceutical preparation is provided. The method involves mixing a pharmaceutically acceptable carrier with a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of

**SUBSTITUTE SHEET**

-5-

isolated heme-containing protein. The preferred aspects are as described above.

According to another aspect of the invention, preparations of improved cell culture medium also are provided. Such preparations are fresh cell culture medium containing a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of isolated heme-containing protein, wherein the gas ligands occupy at least 25% of the heme-moieties. Again, the preferred gas ligands are NO, CO, and CO<sub>2</sub> and the preferred heme-containing proteins are hemoglobin, native hemoglobin, myoglobin, and native myoglobin. The heme-containing protein may be free in solution or contained in liposomes contained in the medium. It is more preferred that the nonoxygen gas ligands occupy at least 50% of the heme moieties, still more preferred that the nonoxygen gas ligands occupy at least 75% of such moieties, and most preferred that the nonoxygen gas ligands saturate the moieties, occupying at least 90% of the moieties. In a particularly preferred embodiment, the fresh, cell culture medium contains isolated, stroma-free, native bovine hemoglobin that is not cross-linked and that is saturated with nonoxygen gas ligand.

The foregoing improved cell-culture media may be prepared by mixing any conventional media with the ligand-bound heme-containing proteins described above.

According, still another aspect of the invention, methods, and products for delivering biological agents are provided. This aspect of the invention involves the use of heme-containing proteins as carriers for delivering biological agents to the environment of cells. The heme-containing protein can have noncovalently bound to them various biological agents, including cell culture enhancers and biologically active molecules. In this manner, such treated heme-containing proteins can be used in the preparation of improved cell culture media or pharmaceutical preparations. They are particularly useful for delivering

SUBSTITUTE SHEET

-6-

non-soluble agents (lipids) and for delivering precise and/or small amounts of biologically active molecules.

The heme-containing proteins, the compositions containing such proteins, and the methods utilizing such proteins can involve any one or all of the delivery of a biological agent, the delivery of a nonoxygen gas ligand, and the delivery of a stabilized oxygen-binding heme moiety. Thus, the preparations of the invention can include fresh cell culture medium or pharmaceutically acceptable carriers mixed with a heme-containing protein that has bound to it one or both of a nonoxygen gas ligand and a biological agent. The invention also provides isolated heme-containing proteins having noncovalently bound to their isolated biological agents, including lipids and cell culture enhancers.

All of the foregoing products may be used in connection with the methods of the invention. As will be readily understood, the environment of the cells can be contacted with the various products described above, including heme-containing proteins that have bound to them one or both of a nonoxygen gas ligand and a biological agent. The preferred heme-containing proteins and concentration of nonoxygen gas ligand are as described above.

#### Brief Description of the Drawings

Fig. 1 is a graph illustrating that the methemoglobin level remains constant throughout the period of addition of carbon monoxide to the bovine hemoglobin isolated as described in the examples below.

Fig. 2 is a graph illustrating cell growth when different doses of the material prepared according to the examples below are provided in tissue culture.

Fig. 3 is a graph illustrating that the presence of hemoglobin prepared according to the examples below results in a decrease in the rate of loss of oxygen from the medium to which the hemoglobin is applied.



-7-

Detailed Description of the Invention

The invention relates, in one important aspect, to loading heme-containing proteins with nonoxygen gas ligands. As described in greater detail below, such gas-carrying proteins are useful for a variety of purposes, including both in vitro and in vivo. Such applications include delivering nonoxygen gas ligands to cells to stimulate metabolic activity and to provide metabolic substrates. They also include providing a stable oxygen trap that enhances cell growth and lengthens the useful life of cell-culture media. The applications also include providing blood substitutes.

As used herein, a "heme moiety" is an iron-containing prosthetic group of a protein. Such proteins are called heme-containing proteins. The heme moiety may be ferrous (II) or ferric (III) and is capable of reversibly binding to the nonoxygen gas ligands of the invention. Preferably, the heme moiety includes a tetrapyrrole ring such as a protoporphyrin (IX) ring. It is preferred that the heme moiety be capable of reversibly binding oxygen, although this is not necessary for all applications herein.

A "heme-containing protein" as used herein is a gas-ligand transporter that preferably is otherwise free of biological activity, such as enzymatic activity. As a result, the heme-containing proteins useful in the invention will not have biologically or medically unacceptable side effects. It will be understood, however, that the heme-containing proteins useful in the invention may have biological activity other than providing a transporter function, provided that such activity does not produce a medically unacceptable side effect or a side effect unacceptable for in vitro use.

As used herein, heme-containing proteins that are gas-ligand transporters reversibly bind oxygen, carbon monoxide, carbon dioxide, or nitric oxide. Specifically excluded are those molecules that release gas ligands as a result of chemical modification of the molecule (e.g.

SUBSTITUTE SHEET

-8-

nitroprusside) as opposed to reversibly binding and releasing a gas ligand.

The gas-ligand transporters useful in the invention include natural transporters, such as native hemoglobin and native myoglobin, isolated from blood or produced synthetically, such as by genetic engineering. As used herein, native hemoglobin and native myoglobin are terms restricted to the foregoing. The native hemoglobins include  $\alpha_2\beta_2$ ,  $\alpha_2\gamma_2$ ,  $\alpha_2\delta_2$ ,  $A_{1a1}$ ,  $A_{1a2}$ ,  $A_{1b}$  and  $A_{1c}$ , as well as any sub-units thereof.

The gas-ligand transporters useful in the invention also include various modifications of the foregoing native transporters, such as those that result from modification by intermolecular and/or intramolecular cross-linking or from complexing with other molecules. As used herein, the terms hemoglobin and myoglobin encompass not only native hemoglobin and native myoglobin, but also the various recombinant derivatives and protein, chemically modified derivatives as described in more complete detail below.

Some other examples of gas-ligand transporters that are heme-containing proteins include the following analogs and derivatives of hemoglobin: (1) 2, 3-diphosphoglycerate (DPG) or DPG analogs such as pyridoxal-5'-phosphate-(PLP) covalently attached to hemoglobin in its deoxy state (Greenberg et al., Surgery, Volume 86 (1979)); (2) polymerized PLP-hemoglobin intermolecularly cross-linked by a nonspecific crosslinker, Bonhard et al., U.S. Pat.No.4,136,093; (3) PLP-hemoglobin conjugated polyethelene glycol, Iwasaki et al., Artificial Organs, Volume 10, No. 5 (1986); (4) hemoglobin conjugated with dextran, Tan et al. Pros. Nat.'l Acad. Sci. U.S.A. Vol. 73 (1976); (5) intramolecularly polymerized hemoglobin, U.S. Pat.No. 4,061,736; (6) hemoglobin conjugated to polyalkylene glycol, Iwashida U.S. Pat.Nos.4,412,989 and 4,301,144; (7) hemoglobin conjugated to polyalkylene oxide, Iwasaki, U.S. Pat.No.4,670,417; (8) hemoglobin conjugated with inositol

-9-

phosphate, Nicolau U.S. Pat.Nos.4,321,259 and 4,473,563; (9) hemoglobin conjugated with an inositol phosphate and a polysaccharide, Wang U.S. Pat.No.4,710,488; (10) hemoglobin conjugated proteins and gelatin derivates, Bonhard, U.S. Pat.No.4,336,248; (11) intramolecularly cross-linked hemoglobin, Walder, U.S. Pat.Nos.4,598,064 and 4,600,531; (12) hemoglobin conjugated with insulin, Ajisaka, U.S. Pat.No.4,377,512; (13) cloned hemoglobin and mutants thereof, Hoffman, U.S. Pat.No.5,028,588; Liebhaver, P.N.A.S. (U.S.A.) 77(1980); and Marotta, et al. J.Biol.Chem., 252(1977) and (14) hemoglobin cross-linked with diaspirin, U.S. Pat.No.4,529,719; (15) BIS-diamide covalently cross linked, pyridoxal-5'-phosphate covalently modified tetrameric hemoglobin, Tye, U.S. Pat. No. 4,529,719; and (16)  $\alpha$ , $\omega$ -dialdehyde cross-linked hemoglobin, Scannon, U.S. Pat. No. 4,473,496.

The foregoing examples are but representative of the various heme-containing proteins known to those of ordinary skill in the art, and the invention is not intended to be restricted to the foregoing list. The entire disclosures of the foregoing patents and references are incorporated herein by reference.

A nonoxygen gas ligand, as used herein, is a molecule that is a gas at room temperature. Such nonoxygen gas ligands include only those ligands that have an electro-negative charge and that are capable of reversibly binding to the heme moiety of the heme-containing proteins used in the invention. Such reversible binding has been well characterized for oxygen, which is specifically excluded from the term nonoxygen gas ligand. The nonoxygen gas ligands include, but are not limited to: carbon monoxide (CO); nitric oxide (NO); and carbon dioxide (CO<sub>2</sub>).

In connection with hemoglobin, which is a tetramer, the dynamics of gas ligand binding are well known to those of ordinary skill in the art. As discussed above, certain aspects of the invention specify that the gas ligand be bound

**SUBSTITUTE SHEET**

-10-

to at least 25% of the heme moieties present. For a molecule such as hemoglobin, that would indicate that at least one heme moiety of the four heme moieties of hemoglobin has bound to it a gas ligand. It is preferred, however, that at least 50% of the heme moieties (even more preferred that at least 75% of the heme moieties) have bound to them a gas ligand. This would correspond to at least two (or three) of the four heme moieties of hemoglobin being saturated. Most preferably, the heme moieties of the heme-containing proteins are completely saturated with nonoxygen gas ligand. Using the detection equipment available to the inventors, readings of about 90% saturation were obtained with material described in the examples below. Nevertheless, it is believed that such molecules are completely saturated and that the readings indicating less than 100% saturated result from limitations relate to the detection equipment, as opposed to the ability to completely saturate the heme moieties.

The methods for saturating the heme moieties of the heme-containing proteins are well known to those of ordinary skill in the art, and typically involve bubbling the desired nonoxygen gas ligand through the solution containing the protein. The resulting proteins are loaded with nonoxygen gas ligand, and the hemoglobin is stabilized against conversion to methemoglobin.

The heme-containing proteins loaded with nonoxygen gas ligand are contacted with the environment of cells. This is a departure from the prior art, which replaced the nonoxygen gas ligand CO with oxygen prior to use. The environment of the cell, when used in connection with in vitro procedures means the fluid or medium in which the cells are suspended or growing. The environment of the cell, when used in connection with in vivo applications, means contacted with a living animal via topical administration, parenteral administration, systemic administration, and the like.

For cell culture, the heme-containing proteins of the invention are mixed with fresh cell culture medium. Fresh

SUBSTITUTE SHEET

-11-

cell culture medium, as used herein, means cell culture medium that has not yet been applied to cells, but rather is of the type that is stored in sterile containers for the intended use with cell culturing. Cell culture media are well known to those of ordinary skill in the art, and include commercially available products such as RPMI-1640, HAM F12, Medium 199, Eagles Minimum Essential Medium, Hybridoma Serum Free Medium, SF900 Insect Cell Medium, Excell 400, Ultra CHO, AIM-V, Keratinocyte SFM, Macrophage SFM, Endothelial SFM, LC-115, and Hybridoma PFHM.

Cell culture medium may be prepared according to procedures well known to those of ordinary skill in the art. In the applications herein, fresh cell culture medium is admixed with a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of heme-containing protein, wherein the gas ligands occupy at least 25% of the heme moieties. Likewise, the fresh cell culture medium may be admixed with heme-containing protein having noncovalently bound to it isolated cell culture enhancers or biological agents. This will be discussed in greater detail below.

As used herein in connection with proteins, agents, and the like, isolated means separated from the native environment in substantially pure form. According to the procedures set forth in the examples below, isolated bovine hemoglobin means bovine hemoglobin that has been separated from red blood cell stroma and red blood cell cytoplasm. The heme-containing proteins of the invention, of course, can be further isolated and purified as described below.

The term isolated used in connection with biological agents, lipids, or cell culture enhancers means isolated separate and apart from hemoglobin and from a source other than red blood cells. Specifically excluded are those preparations of hemoglobin-associated materials, such as superoxide dismutase and carbonic anhydride that tend to co-isolate with hemoglobin prepared for example according to

**SUBSTITUTE SHEET**

-12-

the examples below. Thus, it should be apparent to those of ordinary skill in the art that the term "isolated heme-containing protein having non-covalently attached to it isolated biological agents" specifically excludes those compositions that may occur during the course of isolating hemoglobin from red blood cells. Isolated also refers to recombinantly-derived materials.

The heme-containing proteins loaded with nonoxygen gas ligands also are useful in vivo in treating animal subjects. Preferably the animal subjects are mammals and, most preferably the mammals are humans, primates, horses, cows, dogs, cats, goats, sheep, and pigs. The present invention, thus, involves the use of pharmaceutical formulations which include the heme-containing proteins of the invention together with one or more pharmaceutically acceptably carriers and optionally other therapeutic ingredients. The carrier(s) and other ingredients must, of course, be pharmaceutically acceptable.

In therapeutic applications, the molecules of the invention can be formulated for a variety of modes of administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mac Pub. Co., Easton, Pa.

The particular administration route selected will depend upon the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces therapeutic levels of the heme-containing proteins of the invention, without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, transdermal or parenteral (e.g. subcutaneous, intramuscular, and intravenous) routes. Other routes include intraparenchymal injection into targeted areas of an organ, such as a heart.

SUBSTITUTE SHEET

-13-

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation which is preferably isotonic with the blood of the recipient. Such aqueous preparations may be formulated according to known methods. Among the acceptable vehicles and solvents that may be employed are water, Ringer's Solution, and isotonic sodium chloride solution. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, wetting agents. In addition, detergents may be used to facilitate permeation.

When used as a therapeutic agent, the compositions of the invention are administered in therapeutically effective amounts. A therapeutically effective amount means that amount necessary to delay the onset of, inhibit the progression of, or halt altogether the onset or progression of, the particular condition being treated. Such amounts will depend, of course, on the particular condition being treated, the severity of the condition and individual patient parameters such as age, physical condition, size, weight, and concurrent treatment. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. It will be understood by those of ordinary skill in the art, however, that a patient may insist upon a lower dose or tolerable dose for medical reasons, psychological reasons or virtually any other reason.

When administering the heme-containing proteins of the invention in vivo, issues such as renal toxicity, half-life of the heme-containing protein, and the presence of endotoxins should be addressed. In general, endotoxins are avoided by preparing hemoglobin using sterile techniques; residual endotoxins are then removed by established

SUBSTITUTE SHEET

-14-

techniques. Procedures for accomplishing such preparations are well-known to those of ordinary skill in the art, and include procedures such as that described by: Eichentoph, U.S. Patent 4,526,715; Fesla et al., Surgery, Gynecology and Obstetrics, November 1983, Volume 157, Number 5, p. 399-408; Sheffield et al., Biotechnology and Applied Biochemistry; Volume 9, 1987, 230-238.

To avoid renal toxicity and to extend the half-life of the heme-containing protein, the heme-containing protein can be intermolecularly crosslinked and/or intramolecularly cross linked. The heme-containing protein also can be derivatized with various agents, as well described in the prior art (see the patents and references listed above).

In addition, heme-containing proteins may be incorporated into liposomes. In general, a liposome is a spherical particle of lipid substance suspended in an aqueous medium. The use of liposomes has the additional advantage of protecting the hemoglobin from interaction with the kidney. Liposomes include those with membranes consisting of, but not limited to, hydrogenated phospholipid (U.S. Patent 5,649,391), cholesterol, saturated phosphatidylcholine with an acyl chain in excess of 14 carbons and negatively charged lipid (U.S. Patent 4,911,929), and hydrogenated soy phosphatidylcholine and distearoyl phosphatidylcholine, cholesterol, dimyristoyl phosphatidylglycerol, and alpha-tocopherol (U.S. Patent 4,776,991). The entire disclosures of the foregoing patents are incorporated herein by reference. In addition, other techniques for forming liposomes are well known to those of ordinary skill in the art.

The utilities of the invention are many fold. When used in vitro, the compositions of the invention are useful, for example, in stabilizing heme-containing proteins, in extending the length of the life of culture medium, in enhancing cell growth, in providing metabolic substrates, and in stimulating metabolic activity. When hemoglobin is loaded

SUBSTITUTE SHEET



-15-

with nonoxygen gas ligands and mixed with fresh culture medium it is stabilized both prior to application to the cells (during storage) and after application to the cells (during use). In addition, an important improvement provided by the invention is that cell culture medium containing stabilized hemoglobin will extend the useful life of the cell culture medium. This is demonstrated in the examples below, which show that nearly double the amount of cells can be produced using a fixed quantity of the medium of the invention as compared with prior art medium. Although the inventors do not wish to be bound by any particular theory of the invention, it is believed that the medium may become more useful because: (1) the hemoglobin is stabilized against methemoglobin conversion even after being introduced into the environment of the cells; (2) the heme-containing proteins of the invention act to trap oxygen in the media from escaping to the atmosphere; and (3) the nonoxygen gas ligands may stimulate metabolic activity, thereby enhancing cell growth. We have demonstrated, for example, that there is less loss of oxygen to the atmosphere from cell culture medium that contains the heme-containing proteins loaded with carbon monoxide as compared to cell culture medium that does not contain heme-containing protein loaded with carbon monoxide.

The invention also contemplates loading the heme-containing protein with a gas ligand that can act as a substrate or starter material for cell culture, in addition to enhancing the buffering capabilities of the cell culture medium. It is known that bicarbonate is used both as a buffer and nutrient by living cells. In vitro, bicarbonate can be supplied to cell cultures by adding sodium bicarbonate to the liquid medium or by gasing the cultures with carbon dioxide, a process which readily forms carbonic acid and then bicarbonate ions. The culturing is usually performed in an 'open system' that allows the exchange of gases between the atmosphere and the liquid medium. However, when there is a need to grow cells in a closed environment, a mechanism is

-16-

needed to supply a source of carbon dioxide to cells in a way other than via the atmosphere. Closed environment cell culturing is becoming popular because of the HIV virus and other infectious agents. In addition, closed environments are useful in experimental situations when ready supplies of carbon dioxide are not available, such as aboard spacecraft.

The present invention provides a mechanism for loading a heme-containing protein with carbon dioxide and delivering the bound carbon dioxide directly to the culture medium. Since the carbon dioxide is reversibly bound, the concentration of carbon dioxide provided to the cell culture can be controlled by the amount of hemoglobin dissolved in the medium. Thus, heme-containing proteins loaded with carbon dioxide not only serve to trap oxygen, but also act to provide substrates for cell growth and act to provide a buffering agent to the medium. Provision of cell substrates is particularly important in situations where the medium is serum-free.

The compositions of the invention further have a variety of uses in vivo. First, they provide a more stable heme-containing protein for trapping oxygen already in the blood stream. When ligands such as CO and NO are used, they provide the generalized effect of stimulating metabolic activity. This can be particularly useful in trauma situations when blood substitutes generally are appropriate.

The compositions of the invention further are useful for delivering nonoxygen gas ligands to cells in vivo. Nitric oxide is a vasodilator, a smooth muscle relaxant, a platelet inhibitor, an anti-microbial agent, a modifier of cell adhesion, a modifier of neurotransmission, an enhancer of penile erection, an enzyme regulator, an immune regulator, and a cytotoxic modulator. As a result, it has numerous clinical applications.

NO can be useful in, and not limited to, the treatment of systemic hypertension including malignant hypertension, transient ischemic attacks, cerebral or myocardial ischemia,

SUBSTITUTE SHEET

-17-

coronary insufficiency, intestinal or renal ischemia, peripheral occlusive diseases, congestive heart failure, and angina pectoris. It also can be used to enhance transdermal absorption of pharmaceutical agents due to its vasodilatory capabilities. Thus, the compositions of the invention may be combined with drugs or agents that are delivered transdermally.

Nitric oxide also can be useful in the treatment of biliary colic, esophageal or intestinal spasm, ureteral spasm, and uterine spasm. It further can be useful in the prevention and/or treatment of arteriosclerotic plaques and the prevention of coronary artery occlusion, as well as subsequent prevention of myocardial ischemia and/or infarction. It also may be useful in the adjunctive therapy of bacteria which normally infect humans.

NO also is implicated in the treatment of learning disorders, the treatment of Alzheimer's Disease, and enhancement of memory. It further is implicated as adjunctive treatment for both nonsolid and solid tumors, treatment of autoimmune diseases such as rheumatoid arthritis, lupus, erythematosus, diabetes mellitus and thyroiditis, and prevention of bone marrow, renal, hepatic, and cardiac transplant rejection.

Carbon monoxide recently has been implicated for the same indications as discussed above in connection with nitric oxide.

Heme-containing proteins are purified, endotoxin is removed, and a fluid is added to produce a "blood substitute" that is electrolytically, oncologically, and osmotically compatible with blood to provide the benefits of volume expansion and oxygen transport. The heme-containing protein incorporated into the blood substitute is stabilized intra- and/or intermolecularly by methods such as those described above. Heretofore dextran and albumin have been utilized as volume expanders; they have limited benefit, if any, to enhance oxygen transport and eventual delivery of oxygen to

**SUBSTITUTE SHEET**

-18-

tissue. Blood substitution is indicated in situations of acute blood loss, such as trauma with hemorrhage (surgical, accident-related, war related, etc.) or hemorrhage related to medical disorders, e.g. peptic ulcer disease, variceal bleeds, diverticular bleeding. Blood substitutes could be used for full replacement until bleeding is stanching or conjunction with dextran and/or albumin. Blood substitutes can be used in instances of severe anemia such as the anemia secondary to end-stage renal disease or sickle-cell anemia. Blood substitutes can be administered intravenously.

Still another important aspect of the invention involves the use of heme-containing proteins to deliver biological agents. Biological agents may be difficult to administer to a cell because they are lipids (i.e., nonsoluble) or because they are needed in such small or precise amounts that it is necessary to associate them with another carrier molecule to avoid the loss of the agent during the preparation process or culturing process as a result of the agent, for example, adhering to glass or plastic. Albumin has been frequently used in the prior art as such a carrier of biological agents. According to the invention, heme-containing proteins can be used as such carriers. This is particularly important in cell culture applications when serum-free medium is required. When using serum-free medium cells often do not have the necessary factors for the desired growth or activity (e.g. gene regulation, enhanced cell division, etc.). The media then must be supplemented with the desired cell culture enhancers. Such enhancers may need to be noncovalently bound to the carriers to get them into solution or to deliver them in accurate amounts. Thus, the invention employs heme-containing proteins for this purpose and avoids the need to use recombinantly derived and/or isolated albumin for the same purpose. The invention further contemplates using heme-containing proteins for multiple purposes, including ligand-related purposes, as described above, in addition to biological agent delivery. The preferred biological agents

SUBSTITUTE SHEET

-19-

are cell culture enhancers. Cell culture enhancers mean any molecule that is useful in connection with positively influencing cell culture. Such molecules are well known to those of ordinary skill in the art and include a wide array of agents that are used for a variety of cell culturing purposes. They typically enhance cell viability, growth or metabolism. Cell culture enhancers are commercially available as constituents of cell media and may be purchased separately; enhancers may be added alone to the media or be added in association with another carrier molecule such as albumin. Examples of lipid molecules that can be noncovalently bound to hemoglobin and thus solubilized in medium for cell culture include sterols, fatty acids, glycerols, prostaglandins, leucotrienes, triacylglycerols (triglycerides), and sphingolipids. Particular agents include glucocorticoids such as desoxycorticosterone, 11-desoxycortisol, cortisol, corticosterone, aldosterone, 18-hydroxycorticosterone, and the synthetic glucocorticoids such as triamcinolone acetonide, dexamethasone and prednisolone; androgens such as testosterone, dihydrotestosterone, androsterone and the synthetic analogs stanozolol, danazol, testosterone cypionate, and nandrolone decanoate; estrogens such as estradiol, estrone, estriol and the synthetic estrogens quinestrol and estradiol cypionate; progestins such as progestone, 17-hydroxyprogesterone and the synthetic progestins medroxyprogesterone acetate and megestrol acetate; nonsteroidal compounds such as tamoxifen, clomiphene and diethylstilbestrol; fatty acids including naturally occurring fatty acids and in particular palmitic acid, stearic acid, linoleic acid, oleic acid and arachidonic acid; and phosphacylglycerols such as phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines, and phosphatidylinositol.

In addition to solubilizing nonaqueous materials, heme-containing proteins may be used to bind trace metals such as zinc, nickel, copper, and selenium. They also may be

**SUBSTITUTE SHEET**

-20-

used as a carrier of growth factors such as epidermal growth factor, growth hormone, insulin and fibroblast growth factor. When used in conjunction with trace amounts of mitogenic protein, the heme-containing proteins will prevent nonspecific binding of the mitogens thus effectively maintaining biologically active concentrations of the mitogens.

The biological agent delivering properties of heme-containing proteins also may be used for in vivo applications. Agents useful for in vivo applications are those that are appropriate for the condition being treated. Most preferred are those agents useful in connection with conditions that also require the gas ligand related function of the heme-containing proteins described herein. In addition to the agents described above in connection with in vitro applications, the heme-containing proteins can be used to assist in the delivery of most enzymes and drugs, as will be well recognized by those of ordinary skill in the art.

The present invention also provides for experimental model systems for studying the ability of the heme-containing proteins of the invention to deliver biological agents and enhancers. In these model systems, the biological agents and/or enhancers are noncovalently bound to the heme-containing proteins (which proteins may or may not be loaded with nonoxygen gas ligand). These molecules then are provided to cells via tissue culture medium or to animals in vivo, and the effects are compared to controls which use the same heme-containing protein, but not coated with a biological agent and/or cell culture enhancer. In this manner, methods for discovering useful cell culture medium additives and therapeutics are provided.

#### Example 1

Sterile Collection of Blood: Bovine blood (living donor or slaughterhouse) was collected using sterile procedures and equipment known to those skilled in the art. All containers,

-21-

equipment, and tubing were chemically sanitized or autoclaved before use. Deionized water was used throughout the entire process. Slaughterhouse blood was collected in depyrogenated buckets containing anticoagulant (48.8 mM sodium nitrate • 2 H<sub>2</sub>O, 139 mM sodium chloride, 1.38 mM citric acid anhydrous). Donor blood was collected in plastic bags containing anticoagulant to blood in a 1:4 ratio.

Red Blood Cell Separation: Slaughterhouse blood was transferred to 1.0 liter depyrogenated containers and placed in a Sorval RC-3 refrigerated centrifuge (4°C). When donor blood was used, the blood was collected in donor blood bags, and these bags were placed directly in the centrifuge. The blood was centrifuged at 3000 RPM for 30 minutes. Immediately after centrifugation the plasma and buffy coat was removed aseptically.

Cell Washing: The packed red blood cells were poured through sanitized cheese cloth into a 5-gallon depyrogenated collection tank. The cheese cloth was rinsed with dialysate solution (10 mM sodium phosphate, 200 mM sodium chloride, pH 6.7), and then the red blood cell solution was transferred into a 100-liter processing tank adjusting the volume to 25-30 liters using dialysate solution. The red blood cell solution was diafiltered (cross flow filtration technique) by circulating through a Millipore prostack (16 square feet of membrane, 0.65 µ size) using a Watson-Marlow pump (Model 710R). For 20-26 liters of blood, 180 liters of dialysate buffer are required. Dialysate buffer was pumped into processing tank at an equivalent rate to permeate flow rate in order to maintain reservoir volume (initial starting volume). After washing the red blood cells, cells were concentrated to 18-25 grams of hemoglobin/100 ml. (Hemoglobins concentrations were measured using a CO-Oximeter).

Isolation of Hemoglobin: To the concentrated red blood cell solution 1.5 volumes of lysing solution (10mM sodium phosphate, pH 6.7) was added per volume of concentrated

SUBSTITUTE SHEET

-22-

solution. Osmolarity of solution was 130-160 m Osm. The solution was circulated through a system using a prefilter bypass for 5 minutes. The bypass consisted of a prefilter contained in a stainless steel sanitary holder capable of removing stroma without causing excessive back pressure on the system. Upon completion of prefiltration, lysed solution was circulated through a Millipore prostack. The permeate valve was opened and filtrate was collected (hemoglobin stock solution being approximately 100 g/liter) into a sterile container. To the filtrate was added magnesium chloride (1.0 mM), sodium chloride (25.0 mM), lactose (5.5 mM), and pH of the solution was adjusted to 7.5. Ingredients were mixed for 15 minutes. The final osmolarity was 240-270 m Osm.

Stabilization of Hemoglobin: Hemoglobin was saturated by bubbling carbon monoxide through the solution. The percentage of carbon monoxide bound to hemoglobin was monitored using a CO-Oximeter. Ninety percent saturation was achieved.

#### Example 2

##### Carbon Monoxide Stabilized Hemoglobin:

Hemoglobin was isolated as described above. Carbon monoxide was bubbled into a stirred hemoglobin solution. The percentage of carbon monoxide bound to hemoglobin was measured using a CO-Oximeter. At each of the indicated time points, methemoglobin levels were recorded as well. Referring to Fig. 1, 50% saturation with carbon monoxide was reached after approximately 8 minutes and 90% saturation with CO was reached after approximately 25 minutes. The methemoglobin levels remained constant throughout the 25-minute period.

We also tested the stability of hemoglobin saturated with carbon monoxide for seventy-three days stored at 4°C. There was virtually no conversion to methemoglobin over this period of time.

**SUBSTITUTE SHEET**



-23-

Example 3In Vitro Biological Efficacy:

Insect cells (SF9) were seeded at a density of  $3 \times 10^5$  cells/ml into a 500 ml spinner flask containing 300 ml of SF900 insect cell medium (GIBCO). At time zero (seeding), the medium was then supplemented with hemoglobin saturated with carbon monoxide, the hemoglobin added to final concentrations of 1g/L, 3g/L, and 6g/L. A stock solution having a concentration of approximately 79g/L was used as the starting material and diluted to the various final concentrations. The stock solution was prepared as described above in Example 1. The control medium did not receive any supplementation. Incubation of the insect cells were performed at 28°C. Cell counts were taken approximately every 24 hours. The data, appearing in Fig. 2, are plotted as viable cells per ml. As can be seen from Fig. 2, the cells grown in control media exhibited the least amount of growth and the cells receiving hemoglobin-containing carbon monoxide grew faster and in a dose dependent fashion. For example, at about 100 hours, the concentration of cells in the control medium was about  $2 \times 10^6$  whereas the concentration of cells in media containing hemoglobin at 6g/L was approximately  $4 \times 10^6$ . Likewise, at about 130 hours, the differentials were  $4 \times 10^6$  (control) vs.  $8.25 \times 10^6$  (6g/L).

Example 4Oxygen 'Trapping' by Carbon Monoxide Bound Hemoglobin:

Two T-25cm<sup>2</sup> flasks containing 5 ml of hybridoma serum free medium (HSFM) and  $1 \times 10^7$  AE-1 cells/ml were incubated in air at 25°C for three hours to equilibrate the system. The flasks were designated as control (not supplemented with carbon monoxide-bound hemoglobin) and treated (supplemented) with carbon monoxide bound hemoglobin to a final concentration of 1g/L from a stock concentration of 74g/L. After the incubation period, the flasks were agitated to

-24-

increase the dissolved oxygen levels to 21% (time zero). The dissolved oxygen concentration was then measured over time using a microoxygen probe. The results are shown in Fig. 3. As can be seen, the treated medium contained more oxygen than the untreated medium over the course of time.

The foregoing results were surprising. It was not expected that hemoglobin complexed with carbon monoxide would act as an oxygen trap because it was believed from the teachings of the prior art that the carbon monoxide would continue to occupy the binding sites on the hemoglobin because of the higher affinity of carbon monoxide for such binding sites. Furthermore, because of the known toxicity of carbon monoxide, the results further are surprising. Thus the higher levels of dissolved oxygen and enhanced cell growth were unexpected.

It further is unexpected that the foregoing results were achieved using noncrosslinked, nonpyridoxilated hemoglobin. The art has taught that better results are achieved when the affinity for oxygen is lowered by crosslinking and pyridoxilating the hemoglobin. Thus, the invention in a preferred aspect involves the use of noncrosslinked, nonpyridoxilated native hemoglobin containing the nonoxygen gas ligand. This has advantages in many applications including the reduction in the steps necessary to prepare the product and consequent reduction in use of toxic crosslinking materials and in cost.

Those skilled in the art will be able to ascertain with no more than routine experimentation numerous equivalents to the specific embodiments of the invention described herein. Such equivalents are considered to be within the scope of the invention and are intended to be embraced by the following claims in which we claim:

**SUBSTITUTE SHEET**

CLAIMS

1. A method for delivering a nonoxygen gas ligand to a cell, comprising contacting the environment of the cell with a nonoxygen gas ligand that is reversibly bound to a heme moiety of a heme-containing protein for the purpose of delivering the nonoxygen gas ligand to the cell.

2. A method as claimed in claim 1 wherein the environment of the cell is contacted with a gas ligand selected from the group consisting of NO, CO, and CO<sub>2</sub>; and wherein the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

3. A method as claimed in claim 1 wherein the environment of the cell is contacted with a gas ligand bound to bovine hemoglobin.

4. A method as claimed in claim 1 wherein the gas ligand bound to the heme-containing protein is contained in a liposome.

5. A method as claimed in claim 2 wherein the gas ligand bound to the heme-containing protein is contained in a liposome.

6. A method as claimed in claim 1 wherein the gas ligand bound to the heme-containing protein is contained in a pharmaceutically acceptable carrier.

7. A method as claimed in claim 1 wherein the gas ligand bound to the heme-containing protein is contained in fresh cell culture medium.

8. A method as claimed in claim 1 wherein the heme-containing protein has a plurality of heme moieties and the gas ligand occupies at least 25% of the moieties.

9. A method as claimed in claim 8 wherein the gas ligand occupies at least 50% of the moieties.

10. A method as claimed in claim 8 wherein the gas ligand occupies at least 75% of the moieties.

-26-

11. A method as claimed in claim 8 wherein the gas ligand occupies at least 90% of the moieties.

12. A pharmaceutical preparation comprising a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of isolated heme-containing protein, wherein the gas ligands occupy at least 25% of the heme moieties.

13. A pharmaceutical preparation as claimed in claim 12 wherein the gas ligands are selected from the group consisting of NO, CO, and CO<sub>2</sub>; and wherein the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

14. A pharmaceutical preparation as claimed in claim 12 wherein the gas ligand bound to the heme-containing protein are contained in liposomes.

15. A pharmaceutical preparation as claimed in claim 12 wherein the heme-containing protein has non-covalently attached to it a plurality of isolated biologically active molecules.

16. A pharmaceutical preparation as claimed in claim 12 wherein the heme-containing protein has non-covalently attached to it a plurality of isolated lipid molecules.

17. A pharmaceutical preparation as claimed in claim 12 wherein the gas ligand occupies at least 50% of the moieties.

18. A pharmaceutical preparation as claimed in claim 12 wherein the gas ligand occupies at least 75% of the moieties.

19. A pharmaceutical preparation as claimed in claim 12 wherein the gas ligand occupies at least 90% of the moieties.

20. A method for forming a pharmaceutical preparation comprising

mixing with a pharmaceutically acceptable carrier a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of isolated heme-containing protein, wherein the gas ligands occupy at least 25% of the heme moieties.

-27-

21. A method as claimed in claim 20 wherein the gas ligands are selected from the group consisting of NO, CO, and CO<sub>2</sub>; and wherein the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin and native myoglobin.

22. A method as claimed in claim 20 wherein the gas ligands bound to the heme-containing protein are contained in liposomes.

23. A method as claimed in claim 20 wherein the heme-containing protein has non-covalently attached to it a plurality of isolated biologically active molecules.

24. A method as claimed in claim 20 wherein the heme-containing protein has non-covalently attached to it a plurality of isolated lipid molecules.

25. A method as claimed in claim 20 wherein the gas ligand occupies at least 50% of the moieties.

26. A method as claimed in claim 20 wherein the gas ligand occupies at least 75% of the moieties.

27. A method as claimed in claim 20 wherein the gas ligand occupies at least 90% of the moieties.

28. A preparation comprising

fresh cell culture medium containing a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of isolated heme-containing protein, wherein the gas ligands occupy at least 25% of the heme moieties.

29. A preparation as claimed in claim 28, wherein the gas ligands are selected from the group consisting of NO, CO, and CO<sub>2</sub>; and wherein the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

30. A method as claimed in claim 28 wherein the gas ligand bound to the heme-containing protein are contained in liposomes.

31. A method as claimed in claim 28 wherein the heme-containing protein has non-covalently attached to it a plurality of isolated biologically active molecules.

**SUBSTITUTE SHEET**

-28-

32. A preparation as claimed in claim 28 further comprising an isolated cell culture enhancer bound to the heme-containing protein.

33. A method as claimed in claim 28 wherein the heme-containing protein has noncovalently attached to it a plurality of isolated lipid molecules.

34. A method as claimed in claim 28 wherein the gas ligand occupies at least 50% of the moieties.

35. A method as claimed in claim 28 wherein the gas ligand occupies at least 75% of the moieties.

36. A method as claimed in claim 28 wherein the gas ligand occupies at least 90% of the moieties.

37. A method for forming improved fresh cell culture media, comprising

mixing with fresh cell culture medium a plurality of nonoxygen gas ligands that are reversibly bound to a plurality of oxygen-binding heme moieties of isolated heme-containing protein, wherein the gas ligands occupy at least 25% of the heme-moieties.

38. A method as claimed in claim 37 wherein the gas ligands are selected from the group consisting of NO, CO, and CO<sub>2</sub>; and wherein the heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

39. A method as claimed in claim 37 wherein the gas ligand bound to the heme-containing protein are contained in liposomes.

40. A method as claimed in claim 37 further comprising binding non-covalently a cell culture enhancer to the heme-containing protein.

41. A method as claimed in claim 37 further comprising an isolated biologically active molecule to the heme-containing protein.

42. A method as claimed in claim 37 further comprising binding noncovalently an isolated lipid to the heme-containing protein.

-29-

43. A method as claimed in claim 37 wherein the gas ligand occupies at least 50% of the moieties.

44. A method as claimed in claim 37 wherein the gas ligand occupies at least 75% of the moieties.

45. A method as claimed in claim 37 wherein the gas ligand occupies at least 90% of the moieties.

46. A preparation comprising  
fresh cell culture medium containing isolated heme-containing protein having non-covalently bound to it isolated cell culture enhancers.

47. A preparation as claimed in claim 46 wherein the isolated heme-containing protein is selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

48. A preparation as claimed in claim 46 wherein the cell culture enhancer is a biologically active molecule.

49. A preparation as claimed in claim 46 wherein the cell culture enhancer is a lipid.

50. A method for preparing improved fresh cell culture comprising

mixing with fresh cell culture medium a heme-containing protein that has noncovalently attached to it cell culture enhancers.

51. A method as claimed in claim 50 wherein the fresh cell culture medium is mixed with a heme-containing protein selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin.

52. Isolated heme-containing protein having noncovalently bound to it isolated biological agents.

53. Isolated heme-containing protein having noncovalently bound to it isolated lipid.

54. Isolated heme-containing protein having noncovalently bound to it isolated cell culture enhancers.

55. A method for delivering a biological agent to a cell comprising noncovalently binding the agent to a heme-containing protein and then delivering the heme-containing protein to the environment of the cell.

SUBSTITUTE SHEET

-30-

56. A method as claimed in claim 55 wherein isolated lipid is noncovalently bound to protein.

57. A method as claimed in claim 55 wherein isolated cell culture enhancer is noncovalently bound to the protein.

58. A method for culturing cells, comprising contacting the environment of the cells with a protein containing oxygen-binding heme moieties, wherein at least 25% of the heme-moieties are occupied with a nonoxygen gas ligand.

59. A method as claimed in claim 58 wherein the environment of the cells is contacted with a protein selected from the group consisting of hemoglobin, native hemoglobin, myoglobin, and native myoglobin; and wherein the gas ligand is selected from the group consisting of: NO, CO, and CO<sub>2</sub>.

60. A method as claimed in claim 58 wherein at least 50% of the heme-moieties are occupied with the nonoxygen gas ligand.

61. A method as claimed in claim 58 wherein at least 75% of the heme-moieties are occupied with the nonoxygen gas ligand.

62. A method as claimed in claim 58 wherein at least 90% of the heme-moieties are occupied with the nonoxygen gas ligand.

63. A method as claimed in claim 58 wherein the gas ligands is NO.

64. A method as claimed in claim 58 wherein the gas ligand is CO<sub>2</sub>.

65. A method as claimed in claim 58 wherein a cell culture enhancer is noncovalently bound to the heme-containing protein prior to contacting the environment of the cells with the protein.

66. A method as claimed in claim 65 wherein the biological agent is an isolated lipid.

**SUBSTITUTE SHEET**



1/3

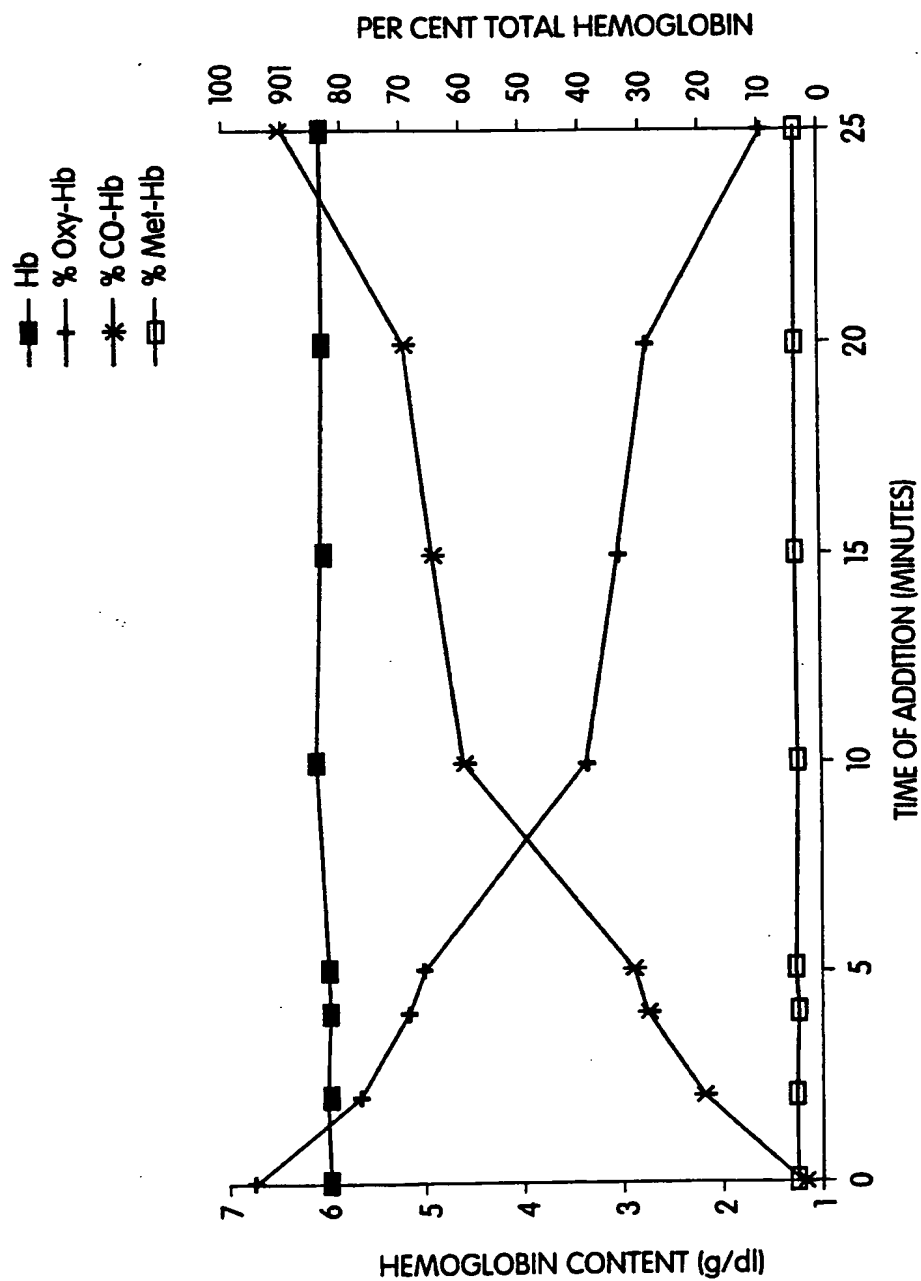


Fig. 1

2/3

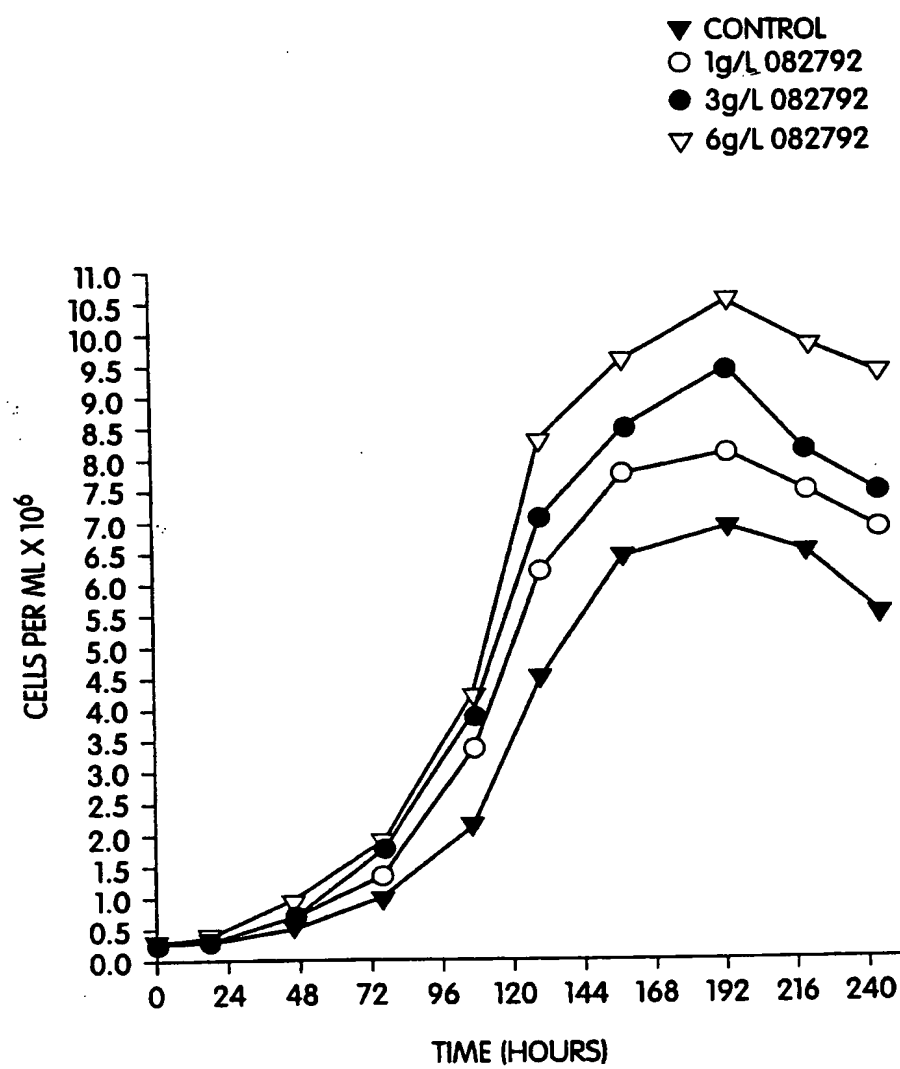


Fig. 2

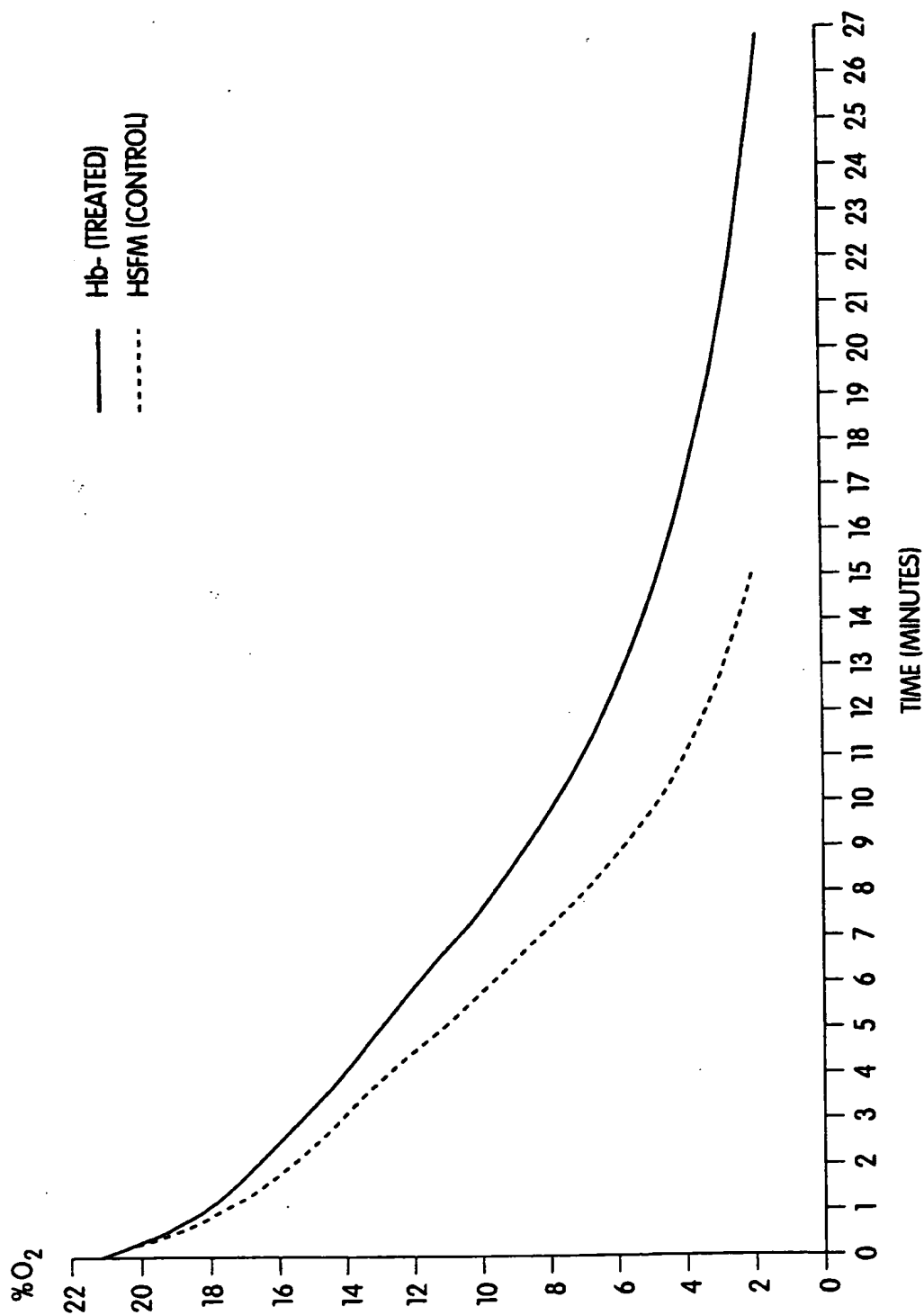


Fig. 3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 94/03116

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 5 A61K47/42 A61K37/14 A61K33/00 C12N5/00 C07K17/02

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,85 04326 (BIOCOMPATIBLES LTD.) 10 October 1985  see page 1, line 1 - line 5; claims 1,8,23 see page 25, line 7 - page 26, line 12 ---	1-6, 8-14, 17-22, 25-27
A	EP,A,0 290 252 (HSIA, JEN-CHANG) 9 November 1988  see page 3, line 57 - page 4, line 1 see page 7, line 54 - page 8, line 47 see claims 1,20-24 --- -/--	1-6, 8-14, 17-22, 25-27

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

19 July 1994

Date of mailing of the international search report

22.07.94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+ 31-70) 340-3016

Authorized officer

Ryckebosch, A

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 94/03116

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>WO,A,93 09806 (BRIGHAM AND WOMEN'S HOSPITAL) 27 May 1993</p> <p>see page 19, line 22 - line 25; claims 13-23,27,35,40-47; example 19</p> <p>see page 20, line 4 - page 22, line 6</p> <p>see page 24, line 10 - line 15</p> <p>see page 25, line 1 - line 5</p> <p style="text-align: center;">----</p>	<p>1-6, 8-14, 17-22, 25-27</p>
P, X	<p>WO,A,93 08842 (SOMATOGEN, INC.) 13 May 1993</p> <p>see page 16, line 11 - line 18; claims</p> <p>see page 28, line 20 - line 26</p> <p>see page 40, line 3 - line 28</p> <p style="text-align: center;">-----</p>	<p>52-55</p>

## INTERNATIONAL SEARCH REPORT

international application No.

PCT/US 94/03116

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark : Although claims 1-6, 8-11, 55, 56 (as far as relating to an in vivo method) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No  
PCT/US 94/03116

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8504326	10-10-85	EP-A, B 0161759 JP-T- 61501513 US-A- 4675310	21-11-85 24-07-86 23-06-87
EP-A-0290252	09-11-88	AU-B- 610883 AU-A- 1563588 CA-A- 1306583 DE-A- 3877811 JP-B- 5032372 JP-A- 63297330 US-A- 4857636 US-A- 5189146	30-05-91 10-11-88 18-08-92 11-03-93 14-05-93 05-12-88 15-08-89 23-02-93
WO-A-9309806	27-05-93	AU-A- 3071592	15-06-93
WO-A-9308842	13-05-93	AU-A- 3132493 AU-A- 3133793 WO-A- 9309143	07-06-93 07-06-93 13-05-93